Bull. Fac. Agr., Saga Univ. (佐賀大農彙) 62 : 1～7 (1987)

# Binding of (−)[$^3$H]Dihydroalprenolol to Erythrocyte Membranes

Takuji TSUKAMOTO, Takashi INABA and Martin SONENBERG*
(Laboratory of Biological Chemistry)
*Received September 24, 1986*

**Summary**

The binding of the β-adrenergic antagonist, (−)[$^3$H]dihydroalprenolol, to isolated membranes has been studied using human erythrocytes. Specific binding of (−)[$^3$H]-dihydroalprenolol to human erythrocyte membranes was temperature- and time-dependent and an equilibrium state of (−)[$^3$H]dihydroalprenolol binding to the membranes could be obtained at a low temperature (5°C). Non-specific binding of (−)[$^3$H]dihydroalprenolol to membranes was less than 20% of the total (−)[$^3$H]dihydroalprenolol binding throughout the equilibrium state. Half-maximal saturation of (−)[$^3$H]dihydroalprenolol binding sites in human erythrocyte membranes occurred at 20 nM (−)[$^3$H]dihydroalprenolol at 5°C. The binding of (−)[$^3$H]dihydroalprenolol was a rapidly reversible process and the half-life of the ligand-receptor complex at 5°C was found to be 3 min. Adrenergic agonist competition studies demonstrated that isoproterenol and epinephrine stereospecifically inhibited (−)[$^3$H]dihydroalprenolol binding to human erythrocyte membranes.

## Introduction

There have been extensive binding studies to identify the β-adrenergic binding sites and/or receptors in turkey[2, 6] and frog[4, 15] erythrocyte membranes using radioactively labelled β-adrenergic antagonists as binding ligands. Results obtained from studies of β-adrenergic antagonist binding in those erythrocyte membranes have largely been in agreement with results obtained from studies of adenylate cyclase stimulation by β-adrenergic agents in time course, specificity and equilibrium dissociation constants (Kd), although binding studies with antagonists may not characterize entirely the interaction of β-adrenergic agonists with β-adrenergic receptors.

Although there has been no direct identification of β-adrenergic binding sites in human erythrocytes, evidence for the existence of β-adrenergic receptors in human erythrocytes has been accumulating[1, 9, 10, 13]. To our knowledge, there is one report regarding the binding of catecholamine to human erythrocytes[3]. In that study, noradrenaline binding to human erythrocytes was shown to be a rapid adsorption process to the surface of the erythrocytes

* Cornell University School of Medical Sciences

rather than specific binding to an adrenergic receptor. In fact, the binding of noradrenaline to human erythrocytes was not influenced by $\alpha$-or $\beta$-adrenergic antagonists[3]. We have previously demonstrated the interaction of $\beta$-adrenergic agonists and antagonists with human erythrocytes[14]. In order to understand this interaction, we have studied the binding of the $\beta$-adrenergic antagonist, (−)[$^3$H]dihydroalprenolol, to isolated human erythrocyte membranes.

## Materials and Methods

**Materials.** (−)-[propyl -2, 3-$^3$H]-dihydroalprenolol ((−)[$^3$H]dihydroalprenolol), 48. 6 Ci/mmol was obtained from New England Nuclear, Boston, Massachusetts. (−) and (+)propranolol were generous gifts from Dr. D. J. Marshall, Ayerst Research Laboratories, Montreal, Canada. Other chemicals were purchased from Sigma Chemical Co., or obtained through Katayama Industries.

**Preparation of erythrocytes and erythrocyte membranes.** Blood was collected directly into heparinized vacutainer tubes from healthy Japanese male blood donors between the ages of 19 and 25 years. The blood was centrifuged at 4°C and washed five times with 10 mM Tris buffer containing 150 mM NaCl (pH7.8). Each erythrocyte preparation was monitored with a cell count of erythrocytes, reticulocytes and leukocytes including a leukocyte differential count. The residual leukocyte count in the erythrocyte preparations was 0.013 ± 0.002% with 0.966 ± 0.021% reticulocytes. Membranes were prepared from washed erythrocytes essentially by a modification[8] of the method of Dodge et al[7].

**(−)[$^3$H]dihydroalprenolol binding assay.** Binding of (−)[$^3$H]dihydroalprenolol to human erythrocyte membranes was determined by a filtration mothod. (−)[$^3$H]dihydroalprenolol (usually 10~30 nM) was incubated with membranes (0.3 mg of membrane protein) for 30 min at 5°C in an incubation medium containing 50 mM Tris buffer (pH 7.4) in a total incubation volume of 0.2 ml. At the end of the incubation, 0.1 ml of incubation medium was diluted with 1.5 ml of cold 50 mM Tris buffer (pH 7.4) and immediately filtered through a Whatman GF/F filter and the filter washed twice with an additional 7 ml of 50 mM Tris buffer (pH 7.4). The filter was dried and the membrane-bound (−)[$^3$H]dihydroalprenolol was determined in 10 ml of scintillation fluid (10 ml of Toluene containing 10% (v/v) BBS-3 solubilizer (Beckman) and 0.4% (w/v) Omniflour (New England Nuclear)) using a Packard 3380 or Aloka LSC-900 liquid scintillation counter. Nonspecific binding is defined as those counts not displaced by excess amounts of non-radioactive (−)propranolol (0.1 mM) and was subtracted from the total (−)[$^3$H]dihydroalprenolol binding to obtain the specific binding.

**Chemical analysis.** Protein was determined by the method of Lowry et al[11].

## Results

**Temperature-dependence of binding.** Specific binding of (−)[$^3$H]dihydroalprenolol

to isolated human erythrocyte membranes was a very rapid and temperature-dependent process. Association of (−)[³H]dihydroalprenolol (12 nM) with erythrocyte membranes reached a maximum within 4~5 min at 5°C (Fig. 1) and 2~3 min at 22°C (Data not shown). At 30°C specific binding of (−)[³H]-dihydroalprenolol to membranes was far more rapid, reaching equilibrium within 30 sec to 1 min, with a subsequent rapid decrease (Data not shown). Thus, because of the difficulty in obtaining equilibrium binding of (−)[³H]dihydroalprenolol to erythrocyte membranes at higher temperatures, a low temperature (5°C) was used as a binding condition throughout these experiments. The non-specific binding of (−)[³H]dihydroalprenolol to membranes was less than 20% of the total (−)[³H]dihydroalprenolol bound during the equilibrium steady state.

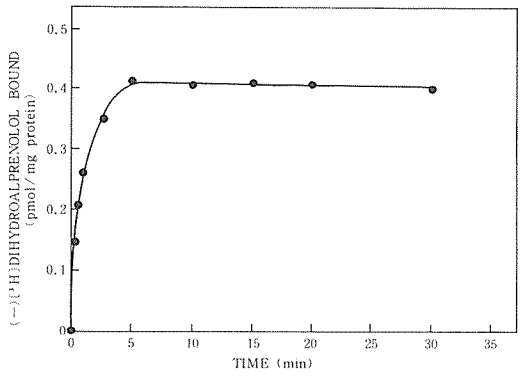


Fig. 1 Specific binding of 12 nM (−)[³H]dihydroalprenolol to human erythrocyte membranes (1.5 mg membrane protein/ml) at 5°C as a function of time.

**Saturation and number of binding sites.** Specific binding of (−)[³H]dihydroalprenolol to human erythrocyte membranes was a saturable process. The half-maximal saturation of (−)[³H]dihydroalprenolol binding sites in the human erythrocyte membrane occurred at 20 nM (−)[³H]dihydroalprenolol (Fig. 2). At saturation, there were 0.96 pmole of (−)[³H]dihydroalprenolol bound per mg membrane protein. The saturation value corresponds to 330 binding sites per cell.

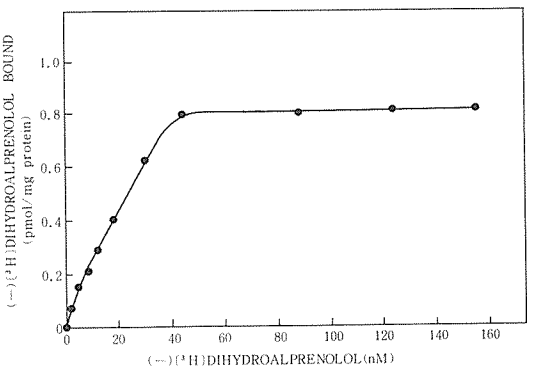


Fig. 2 Specific binding of (−)[³H]dihydroalprenolol to human erythrocyte membranes (1.5 mg protein/ml) as a function of increasing concentration (−)[³H]dihydroalprenolol at 5°C.

**Reversibility of (−)[³H]dihydroalprenolol binding.** Specific binding of (−)[³H]dihydroalprenolol to human erythrocyte membranes was a rapidly reversible process. The half-life of (−)[³H]dihydroalprenolol-receptor complex at 5°C was found to be 3 min (Fig. 3). With temperatures higher than 5°C, it was difficult to determine accurately the half-life of the ligand-receptor complex due to the immediate dissociation of bound (−)[³H]dihydroalprenolol from membranes after dilution of (−)[³H]dihydroalprenolol-membrane complex. Further, the presence of (−)propranolol increased the rate of (−)[³H]dihydroalprenolol dissociation from the membranes, as shown in Fig. 3.

**Stereospecificity of (−)[$^3$H]dihydroalprenolol binding.** Studies of the stereospecificity of (−)[$^3$H]dihydroalprenolol binding were carried out by competitive binding studies of (−)-[$^3$H]dihydroalprenolol with (−) and (+)propranolol (Fig. 4). As shown in Fig. 4, (−)propranolol was found to be more potent than (+)-propranolol in competing for (−)[$^3$H]dihydroalprenolol binding sites in human erythrocyte membranes.

**Agonist competition of (−)[$^3$H]dihydroalprenolol binding.** Competitive binding of (−)-[$^3$H]dihydroalprenolol to human erythrocyte membranes with the (−) and (+)$\beta$-adrenergic agonists of isoproterenol, epinephrine and norepinephrine is shown in Fig. 5. The most effective agonists in this regard were (−)isoproterenol and (−)epinephrine which yielded similar competition curves. Half-maximal inhibition of total binding of (−)[$^3$H]dihydroalprenolol by (−)isoproterenol was noted at approximately 90 $\mu$M while that of (+)isoproterenol was about an order of magnitude less effective with a Kd of 0.8 mM. Half maximal competition of (−)[$^3$H]-dihydroalprenolol binding was not realized with (−) or (+)norepinephrine, even with concentrations as high as 1 mM, and the same was true for (+)epinephrine.

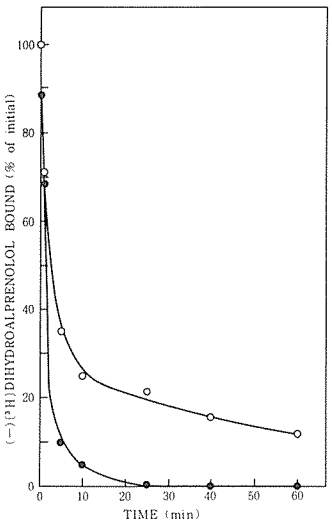


Fig. 3 Rate of dissociation of specifically bound (−)[$^3$H]dihydroalprenolol. Human erythrocyte membranes (1.9 mg/ml) and 25 nM (−)[$^3$H]dihydroalprenolol were incubated, and after 20 min at 5°C, the reaction mixture was diluted (100 fold) into the same buffer in the presence or absence of unlabelled 50 $\mu$M (−)propranolol. At the intervals indicated at 5°C, residual (−)[$^3$H]dihydroalprenolol bound was determined. (t=0 refers to the sampling time immediately after 100-fold dilution). ○, dissociation in the absence of added (−)propranolol; ●, dissociation in the presence of (−)propranolol (50 $\mu$M).

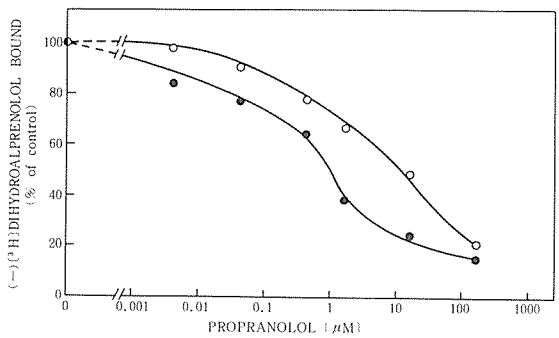


Fig. 4 The stereospecific inhibition of total (−)-[$^3$H]dihydroalprenolol binding to human erythrocyte membranes by non-radioactive propranolol. The binding was initiated by addition of membranes to the reaction mixtures already containing 12 nM (−)[$^3$H]dihydroalprenolol and non-radioactive (−) or (+)propranolol, (○) and (●), respectively.

Moreover, the $\alpha$-adrenergic antagonist, phentolamine, had no effect on the (−)[$^3$H]dihydroalprenolol binding over the concentration range (0.1 $\mu$M~0.1 mM) used here.

## Discussion

Our results demonstrate the existence of specific (−)[$^3$H]dihydroalprenolol binding sites in human erythrocyte membranes. Association of (−)[$^3$H]dihydroalprenolol with human erythrocyte membranes was a very rapid

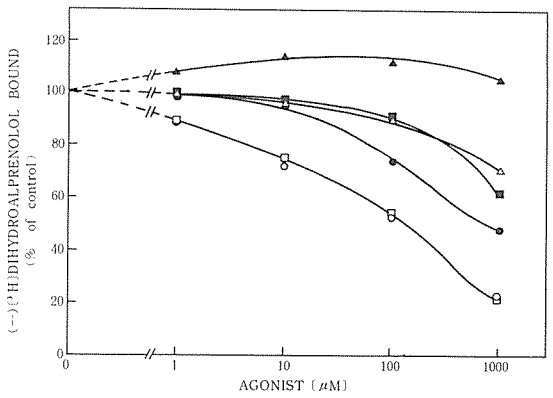


Fig. 5 Competition for total (−)[³H]dihydroalprenolol binding sites in human erythrocyte membranes by adrenergic agonists. Refer to Legend to Fig. 4. The adrenergic agonists used in this study were (−) and (+)isoproterenol (○, ●), (−) and (+)epinephrine (□, ■) and (−) and (+)norepinephrine (△, ▲).

temperature-dependent process which reached maximal binding within 4～5 min at 5°C (Fig. 1). The binding of (−)[³H]dihydroalprenolol was a rapidly reversibly process. As shown in Fig. 3, even at 5°C, dissociation of (−)[³H]dihydroalprenolol occurred rapidly after dilution (100 times) with buffer. The half-life of the hormone-receptor complex at 5°C was found to be 3 min. The binding of (−)[³H]dihydroalprenolol to human erythrocyte membranes was a stereospecific (Fig. 4) and saturable (Fig. 2) process. The half-maximal saturation of (−)[³H]dihydroalprenolol binding sites in human erythrocyte membranes occurred at 20 nM (−)[³H]dihydroalprenolol at 5°C (Fig. 2). This apparent Kd obtained from the saturation study is comparable to the Kd noted with intact frog erythrocytes[12].

It has been reported that no binding of (−)[³H]dihydroalprenolol to human erythrocytes is detectable[15]. However, in their assay for (−)[³H]dihydroalprenolol binding to human erythrocytes, 12 min at 37°C were the incubation conditions. We also were unable to detect binding of (−)[³H]dihydroalprenolol to human erythrocyte membranes at these conditions. This is likely to be the result of a rapid decrease in binding after reaching equilibrium within 1 min at 37°C.

Our results cannot be accounted for by contamination by reticulocytes and/or leukocytes. In our human erythrocyte preparation (before hemolysis), no reticulocytes were detectable. Even with 0.1% reticulocytes in our erythrocyte preparation, binding of (−)-[³H]dihydroalprenolol to erythrocyte membranes was the same as with membranes isolated from erythrocytes with no detectable reticulocytes. Moreover, 0.02% or less contamination of leukocytes in our erythrocyte preparation could not account for binding of (−)-[³H]dihydroalprenolol to human erythrocyte membranes. Thus, it is evident that there are specific binding sites for (−)[³H]dihydroalprenolol in human erythrocyte membranes.

Although β-adrenergic agonist could inhibit stereospecifically the binding of (−)-[³H]dihydroalprenolol to human erythrocyte membranes, the Kd values of the β-adrenergic binding to the membranes obtained from the studies were not compatible with that of the β-adrenergic effect on membrane protein kinase[14]. For example, the Kd values for (−)isoproterenol and (−)epinephrine obtained from the (−)[³H]dihydroalprenolol binding studies using the equation previously described[5] were identical, being 90 μM (Fig. 5). On the other hand, the apparent Kd values for (−)isoproterenol and (−)epinephrine obtained from the stimulation of membrane protein kinase were 0.17 μM and 0.35 μM, respectively. This may possibly be due to the partial occupancy of β-adrenergic antagonist

binding sites by β-adrenergic agonist or the reverse.

## Acknowledgements

The authors gratefully acknowledge the kind cooperation of Dr. Yoshifumi Harada, Ms. Miyako Shimaya and Ms. Satoe Ichikawa of the Health Center for drawing blood from donors.

We are very grateful to Ms. Tae Kan of the National Hospital of Saga for her valuable suggestions in leukocyte and reticulocyte countings.

We also thank Mr. Hiroshi Shimizu and Ms. Margaret Priddle for contributing to part of this work.

This work was supported in part by Grants-in-Aid for Scientific Research from the Ministry of Education, Science and Culture, as well as grants from the National Institutes of Health, U. S. A.

## References

1. Allen, E. J. and H. Rasmussen (1971). *Science*, **144**, 512～514.
2. Andre, C., G. Vauquelin, Y. Seerne, J. P. De Backer and A. D. Strosberg (1982). *Biochem. Pharmacol.*, **31**, 3657～3662.
3. Blust, R. and H. Bamaschak (1976). *Acta Biol. Med. Germ.*, **35**, 709～713.
4. Brandt, D. R. and E. M. Ross (1986). *J. Biol. Chem.*, **261**, 1656～1664.
5. Cheng, Y. C. and W. H. Prussott (1973). *Biochem. Pharmacol.*, **22**, 3099～3108.
6. Citri, Y. and M. Schramm (1982). *J. Biol. Chem.*, **257**, 13257～13262.
7. Dodge, J. T., C. D. Mitchell and D. J. Hanahan (1963). *Arch. Biochem. Biophys.*, **100**, 119～130.
8. Hanahan, D. J. and J. E. Ekholm (1974). *Methods in Enzymol.*, **30**, 168～172.
9. Heustis, W. H. and H. M. McConnell (1974). *Biochem. Biophys. Res. Commun.*, **57**, 478～483.
10. Kaiser, G., K. Quiring, D. Ganger, D. Palm, H. Becker and N. Schoeppe (1974). *Blut.*, **29**, 115～122.
11. Lowry, O. H., N. J. Rosebrough, A. I. Farr and R. J. Randall (1951). *J. Biol. Chem.*, **193**, 265～275.
12. Mukherjee, C. and R. J. Lefkowitz (1976). *Life Sciences*, **19**, 1897～1906.
13. Rodan, S. B., G. A. Rodan and J. I. Sha' afi (1976). *Biochem. Biophys. Acta*, **428**, 509～515.
14. Tsukamoto, T. and M. Sonenberg (1979). *J. Clin. Invest.*, **64**, 534～540.
15. Williams, L. T., R. Snyderman and R. J. Lefkowitz (1976). J. Clin. Invest., **57**, 149～155.

# 赤血球膜への(−)[$^3$H]dihydroalprenololの結合

塚本卓治・稲葉　喬・Martin Sonenberg*
(生物化学研究室・*コーネル大学医学部)

昭和61年9月24日　受理

## 摘　　要

ヒト赤血球から単離した細胞膜への(−)[$^3$H]dihydroalprenolol (β-アドレナリンアンタゴニスト)の結合を調べた．ヒト赤血球膜への(−)[$^3$H]dihydroalprenololの特異的結合は，温度および時間依存性であった．膜への(−)[$^3$H]dihydroalprenolol結合の平衡状態は低い温度(5°C)にて得ることができた．平衡状態を通して，(−)[$^3$H]dihydroalprenololの膜への非特異的な結合は全結合の20%以下であった．ヒト赤血球膜の(−)[$^3$H]dihydroalprenolol結合部位の半分を占有するのに必要な(−)[$^3$H]dihydroalprenololの濃度は5°Cで20 nMであった．(−)[$^3$H]dihydroalprenololのヒト赤血球膜への結合は迅速な可逆過程であり，その〝リガンド-レセプター複合体〟の5°Cにおける半減期は3分であった．さらに，β-アドレナリンアゴニストの存在下において(−)[$^3$H]dihydroalprenololのヒト赤血球膜への結合を調べた結果，isoproterenolとepinephrineが(−)[$^3$H]dihydroalprenololの結合を立体特異的に阻害することを見い出した．